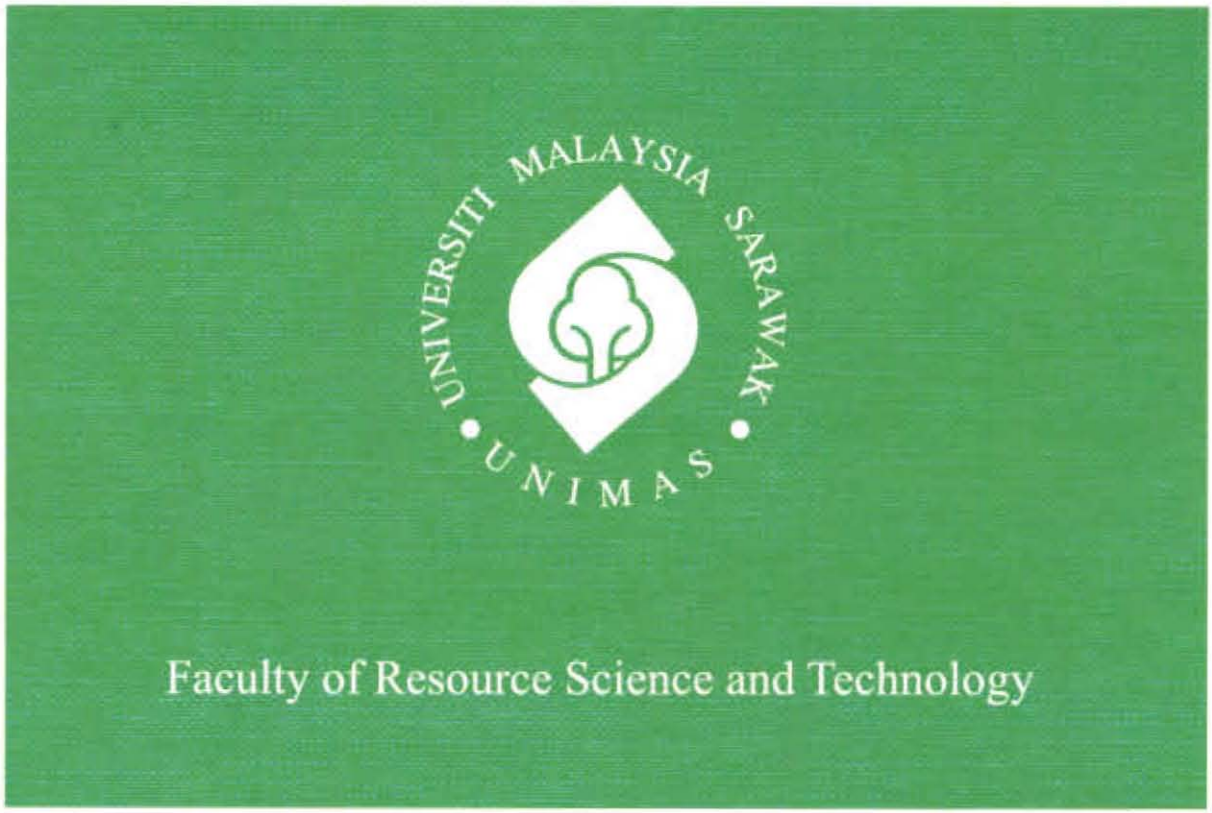

DESIGNING AND TESTING OF PRIMERS FOR CLONING OF *Cinnamoyl-CoA Reductase (CCR)* GENOMIC SEQUENCE FROM KELAMPAYAN *(Neolamarckia Cadamba)*

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Designing and Testing of Primers for Cloning of *Cinnamoyl-CoA reductase* (*CCR*) genomic sequence from Kelampayan (*Neolamarckia cadamba*)

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Designing and Testing of Primers for Cloning *Cinnamoyl-CoA reductase* (*CCR*) genomic sequence from Kelampayan (*Neolamarckia cadamba*)

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Abstract

Lignin content in the wood is an important criterion to choose planting materials for economic purposes. A study of one of the lignin biosynthesis genes, *cinnamoyl-CoA reductase* (*CCR*) from *Neolamarckia cadamba* was carried out. Partial *CCR* DNA fragment was amplified by using the primer set 1 designed from partial cDNA of *Neolamarckia cadamba*. The amplification product was then examined on 1.5 % agarose gel and continued with the purification of the product. The purified product was then cloned into pGEM®-T Easy Vector. One positive clone was sent for sequencing and the data obtained was analyzed. The amplified product using primer set 1 did not show homology to the *CCR* gene from *Neolamarckia cadamba*. Therefore, primer set 2 was designed from partial cDNA of *Neolamarckia cadamba* and tested to amplify the target region of the *CCR* gene. Polymerase chain reaction (PCR) optimization of *CCR* gene from *Neolamarckia cadamba* was performed by using primer set 2. The PCR product was then purified and cloned into pGEM®-T Easy Vector. However, the estimated product size of the colony PCR of the *CCR* was not observed positive clone was not obtained. Few solutions could be taken in order to successfully clone the desired PCR product into the pGEM®-T Easy Vector.

Key Words: *Neolamarckia cadamba*, *Cinnamoyl-CoA reductase* (*CCR*), Polymerase Chain Reaction (PCR), primer set

Abstrak

Kandungan lignin di dalam kayu ialah satu kriteria penting untuk memilih pokok yang dapat mengaut keuntungan. Jujukan DNA bagi gen, cinnamoyl-CoA reductase (CCR) daripada Neolamarckia cadamba telah dikaji. Jujukan CCR DNA telah diamplifikasikan dengan menggunakan set pencetus 1 direka daripada segmen cDNA Neolamarckia cadamba. Hasil amplifikasi segmen DNA diklonkan ke dalam pGEM®-T Easy Vector dan satu positif klon dihantar untuk penjujukan. Hasil analysis data tersebut menunjukkan hasil amplifikasi tersebut tidak berhomolog dengan jujukan CCR DNA daripada Neolamarckia cadamba. Oleh itu, set pencetus 2 direka daripada segmen cDNA Neolamarckia cadamba dan diuji untuk mengamplifikasikan jujukan CCR DNA. Optimisasi PCR gen CCR dari Neolamarckia cadamba telah dijalankan dengan menggunakan set pencetus 2. Hasil PCR telah dipurifikasi dan diklonkan ke dalam pGEM®-T Easy Vector. Saiz produk koloni PCR yang dijangka tidak dicapai dan positive klon tidak didapati. Beberapa cara penyelesaian pengklonan dapat digunakan untuk mengklonkan produk PCR yang dikehendaki ke dalam pGEM®-T Easy Vector.

Kata Kunci: Neolamarckia cadamba, Cinnamoyl-CoA reductase (CCR), PCR, set pencetus

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LIST OF ABBREVIATIONS

cDNA	Complementary DNA
CCR	*Cinnamoyl-CoA reductase*
DNA	Deoxyribonucleic acid
GAS	Gene Assisted Selection
LD	Linkage Disequilibrium
MAS	Marker Assisted Selection
mRNA	Messenger RNA
ORF	Open Reading Frame
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase Chain Reaction
QTL	Quantitative Trait Loci
QTNs	Quantitative Trait Nucleotides
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism

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CHAPTER I

INTRODUCTION

Forest trees contribute to the majority of terrestrial biomass production and are vital component of the biodiversity. Current global demand for wood is unable to be compensated by some of the slow growing species forest trees. Thus, this results in the loss and degradation of forest. Fast growing species in the plantation forests such as *Neolamarckia cadamba* have the capability to supply the bulk of wood needs on a long-term basis thus reduce the harvest pressure on natural forest for wood production to an acceptable level.

SARAWAK FORESTRY Corporation is stepping up mass production of genetically improved seeds to meet the growing demand for plantation timber projects. The corporation had set up 11 seed production areas statewide. It is producing seeds of priority species like kapur bukit, meranti sarang punai, kelampayan, benuang and belian on natural stands. The seed orchard would help mass production of quality seeds for large-scale plantation programs. "The government's planted forest project started 11 years ago, targeting to establish a sustainable source to supply the region's timber processors and the state's planned pulp and paper industry", said by the managing director of SARAWAK FORESTRY Corporation, Datuk Len Talif Salleh, during a seed orchard establishment and plus-tree selection workshop at Semenggoh in November 2008.

Neolamarckia cadamba or locally known as Kelampayan is an important fast growing species used in veneer and plywood production. Kelampayan, under the family of Rubiaceae is characterized as a large, deciduous and economically significant species as it provides early economic returns within 8 to 10 years (Joker, 2000). Kelampayan is one of the light-colored timbers with low density which serves as raw material for pulp and paper industry and it is

one of the best materials for plywood industry. According to Ho *et al.* (2009), Kelampayan has been identified as one of the potential fast growing species for planted forest development in Sarawak.

Based on the research done by Lim *et al.* (2005), at Forest Research Institute Malaysia, "The tree is medium sized to large trees (40 m to 45 m tall). Its bole is straight and cylindrical. It is often branchless of up to 25 m and a diameter of up to 100 cm with buttresses to 2 m tall. It is distributed in lowland to mountain forests to 1000 m altitude; often by streams and rivers as well as open sites in the forest". Kelampayan timber is white with coarse surface due to the presence of large vessels. The timber is usually used for light-weight purposes such as picture frames, moulding, skirting, disposable chopstick, wooden sandals, general utility furniture, veneer and plywood as the timber is soft and light.

Lignin is one of the world's most abundant natural polymers, other than cellulose and chitin. Lignin, a phenolic polymer which usually derived from phenylalanine serves as a matrix around the polysaccharides components of the plant cell walls. Lignin is believed to contribute compressive strength, provide additional rigidity, resistance to degradation by microbial attack, rendering the walls hydrophobic and water impermeability to the polysaccharide-protein matrix of the cell wall.

Due to economic purposes, lignin has become one of the criteria for choosing planting material. According to Chiang (2005), tremendous effort has been devoted to developing genetically engineered trees, with the emphasis on reducing lignin quantity to improve wood pulp production efficiency. Yet, lignin chemical reactivity also is a critical barrier to wood pulp production because lignin removal from wood is either initiated by chemical degradations or in most cases, accomplished entirely by chemical reactions. Therefore, the current tree biotechnology emphasis on low lignin quantity must be expanded to include greater lignin reactivity and, ultimately, a combination of low and reactive lignin traits.

In the lignin biosynthesis pathway, many genes such as *cinnamyl alcohol dehydrogenase* (*CAD*), *cinnamate 4-hydroxylase* (*C4H*), *phenylalanine ammonia-lyase* (*PAL*) and others are involved. Nevertheless, in this study, the main focus is *cinnamoyl-CoA reductase* (*CCR*) from Kelampayan trees. Function of *CCR* is to catalyze the reduction of hydroxycinnamoyl CoA esters into cinnamaldehydes in the lignin biosynthesis pathway (Hahlbrock and Scheel, 1989; Lacombe *et al.*, 1997; Lauvergeat *et al.*, 2002).

Goffner *et al.* (1994) hypothesized that *CCR* plays a key regulatory role in lignin biosynthesis as the first committed step in the production of monolignols from phenylpropanoid metabolites. Trees altered in their lignin profile either with reduced amounts of lignin or with a more extractable syringyl-rich composition are more desirable for pulping. Less severe pulping conditions are required for more rapid delignification. This result in decreased of cellulose degradation, reduced chemical consumption and higher pulp yield and strength (Hibberd *et al.*, 1999).

To date, the lignin biosynthesis gene, *CCR* from *N. cadamba* has not been characterized and studied. Hence, a study with the objectives of designing and testing of primers for cloning of *CCR* genomic sequence from *N. cadamba* was conducted. The *CCR* gene was amplified, purified, cloned and sequenced accordingly. Two set of primers were designed for the purpose of PCR. The first primer set and second primer set were designed by using Primer Premier 5.0 software and Primer 3.0 respectively. The positive clone was sent for sequencing. Sequenced result was then analyzed with BLASTn (Altschul *et al.*, 1990) through BLAST Search Engines (http://blast.ncbi.nlm.nih.gov/) to verify the identity of the nucleotide sequence.

CHAPTER II

LITERATURE REVIEW

2.1 *Neolamarckia cadamba* (Kelampayan) trees

Neolamarckia cadamba is distributed in the Asia-Temperate such as China and Asia-Tropical country like India, Nepal, Sri Lanka, Cambodia, Laos, Myanmar, Thailand, Vietnam, Indonesia, Malaysia and Papua New Guinea. Its economical value is environmental, materials and medicines. *N. cadamba* is grown for ornamental purpose and it is potential for agro forestry. The timber can be used in producing fiber and wood. The barks and leaves are reported to possess various medicinal values such as astringent anti-hepatotoxic (Kapil *et al.*, 1995), antidiuretic, antiseptic, wound healing and anthelmintic. Figure 2.1 shows the structures of *Neolamarckia cadamba* tree's bark, leaves and flowers.

(a) (b)

Figure 2.1 *Neolamarckia cadamba* (a) tree's bark (b) leaves and flowers structures. Source: http://prothom-aloblog.com/users/base/computer/146

According to Patel & Kumal (2008) in the pharmacognostical studies of *N. cadamba*, the shape of *N. cadamba* leaves is broadly ovate, elliptic-oblong with entire margin, pulvinus base, bitter in taste, mucronate apex, glabrous surface, pinnate venation, length ranged from 7.5 to 18 cm and breath is 4.5 to 16 cm. Through the microscopic studies, the leaf is dorsiventral with thick prominent midrib and uniformly thin lamina. Ground tissue of the midrib contains a broad outer zone of collenchymas and inner and middle zone of parenchyma. Vascular bundle built up of a horseshoe shape inverted vascular strands and two small top-shaped strands placed at the ends of the 'U'-shaped strands. The epidermis of the midrib is distinct with squarish thick-walled cells and prominent cuticle. On the other hand, microscopic powder of *N. cadamba* showed the presence of unicellular, ligified trichomes, paracytic stomata, simple starch grains and sandy balls of calcium oxalate crystals.

2.2 Lignin Biosynthesis Pathway

Lignins are polymers of three alcohol monomers, or also known as monolignols such as coumaryl alcohol, coniferyl alcohol and sinapyl alcohol that represent coumaryl units (H units), guaiacyl subunits (G units) and syringyl units (S units) respectively. In forming a lignin polymer, each monolignol will form several types of bonds with other monolignol through dehydrogenative polymerization. Lignin content and composition vary between the major groups of higher plants and between species (Freudenberg, 1959 and 1965). For example, angiosperm lignin consist majority of G units and S units.

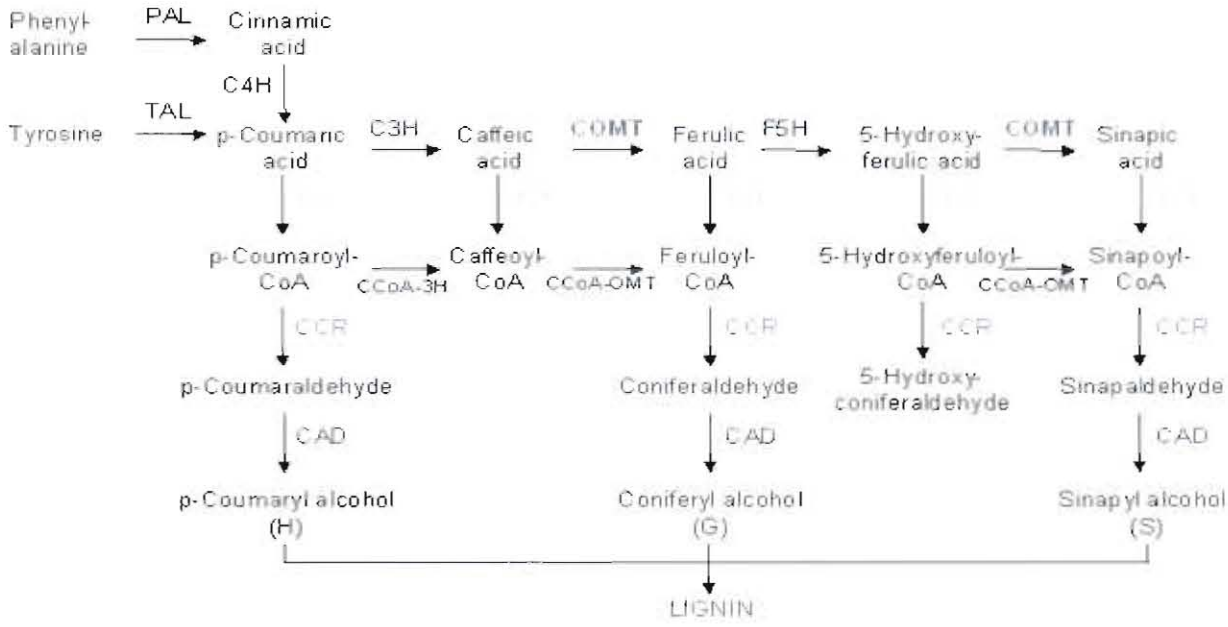

Figure 2.2 Lignin Biosynthesis Pathway (Spangenberg *et al.*, 2001).

Enzymes that catalyze the biosynthetic pathway of lignins belong to the common phenylpropanoid pathway which begins with deamination of phenylalanine and leading to the synthesis of hydroxycinnamoyl CoA esters. Hydroxycinnamoyl CoA esters are drawn towards lignin synthesis through two enzymes. *Cinnamoyl CoA reductase* (*CCR*) reduces hydroxycinnamoyl CoA esters into cinnamaldehydes while *cinnamoyl alcohol dehydrogenase* (*CAD*) catalyzes the reduction of cinnamaldehydes into hydroxycinnamoyl alcohols, the precursors of lignin (Hahlbrock and Scheel, 1989; Lacombe *et al.*, 1997; Lauvergeat *et al.*, 2002).

Secondary xylem (wood) is built up of cellulose, lignin and hemicelluloses. Cellulose microfibrils are embedded in lignin and a hemi-cellulose matrix, which gives strength to the wood tissue (Plomion *et al.*, 2001). Relatively few plant cell types accumulate substantial amounts of lignin during normal development. Xylem elements and sclerenchyma cells that

accumulate lignin usually have other cell wall modifications, for example secondary cell wall thickening that plays the same role in strengthening the cell. Genes participated in lignin biosynthesis respond to both developmental and environmental signals as disease or wounding can induce lignin deposition in many cell types (Whetten and Sederoff, 1995).

2.3 *Cinnamoyl CoA reductase* (*CCR*) gene

CCR catalyzes the reduction of hydroxycinnamoyl-CoA thioesters to the corresponding aldehydes in the lignin biosynthesis pathway. *CCR* gene in *Eucalyptus urophylla* co-lacalised with a quantitative trait loci (QTL) for both lignin content (accounting for 13.5% of the phenotypic variation in this trait) and the S:G ratio (24.7%) (Gion *et al.*, 2001). According to Piquemal *et al.* (1998), this information was consistent with results obtained from transgenic studies, with the down regulation of *CCR* to produce a reduction in lignin content in tobacco.

Analysis of natural mutant in *Arabidopsis* has shown that mutations in *cinnamoyl CoA reductase* (*CCR*). a key lignin gene, affect physical properties of the secondary cell wall such as stiffness and strength of wood. According to Thumma *et al.* (2005), using *CCR* gene, tested whether linkage disequilibrium (LD) mapping could identify alleles associated with microfibril angle (MFA). MFA is known as a wood quality trait affecting stiffness and strength of wood. Further analysis of the mutant has shown that alternative splicing in *CCR* gene is responsible for the changes in physical properties.

Twenty-five common single nucleotide polymorphism (SNP) markers in the *CCR* gene in *Eucalyptus nitens* were identified by Thumma *et al.* (2005). Two haplotypes significantly associated with MFA were found by using single-marker and haplotype analyses in 290 trees

from *E. nitens* natural population. These results were confirmed in two full-sib families of *E. nitens* and *E. globulus*. cDNA clones were sequenced and an alternatively spliced variant from the significant haplotype region was identified to understand the functional significance of the SNP markers.

CHAPTER III

MATERIALS AND METHODS

3.1 Collection of Plant Material and DNA samples

The leaf samples of *N. cadamba* used in this study were collected from Kelampayan Trial Plot, Landeh Forest Reserve, Semengoh.

3.2 DNA Isolation and Purification

3.2.1 CTAB Extraction Buffer Preparation

100 ml of 1 M Tris HCl pH 8.0 and 40 ml of 0.5 M EDTA pH 8.0 were mixed. 600 ml of distilled deionised water was added. 81.82 g of 1.4 M NaCl was weighted and added. The volume of mixture was adjusted to 1 L with deionised water, autoclaved and stored at room temperature. 20 g of 2% Cetyl trimethyl ammonium bromide (CTAB) was weighted and added into the mixture. It was stirred vigorously on a magnetic stirrer for several hours or overnight to dissolve. 10 g of 1% Polyvinylpyrrolidone (PVP) was weighted, added and stirred vigorously on a magnetic stirrer for several hours to dissolve. 40 µl of 2% (v/v) β-mercaptoethanol was added to 20 ml EB immediately before use.

3.2.2 Modified DNA Isolation Protocol

The CTAB DNA isolation method used was modified from the Doyle and Doyle protocol (1990). Initially, water bath was filled with distilled water and the temperature was set at 65°C. Then, 0.8g to 1.0 g of leaf sample was prepared. 4 ml of extraction buffer and 80 µl of β-mercaptoethanol (suggested for small DNA volume) were put into a Falcon tube and were incubated for 30 minutes at 65°C water bath. The leaf sample was washed with water and wiped with 70% ethanol. Then, it was torn into small pieces. The leaf's vines were removed and then put into the pestle. The mortal and pestle must be pre-chilled with liquid nitrogen before grinding. Liquid nitrogen was then added and the leaf sample was grind into powder.

The leaf powder sample was then added into the falcon tube that contained extraction buffer and β-mercaptoethanol. Then, the falcon tube was incubated for 30 minutes with shaking to mix the content. 600 µl of the sample was taken with the cut tip pipette. Next, 600 µl of Chloroform-Isoamyl (CIA) solution was added into each microcentrifuge tube. The microcentrifuge tubes were inverted 25 times and then centrifuged at 13,000 rpm for 10 minutes. The upper aqueous phase was transferred to a new microcentrifuge tubes and the CIA extraction step was repeated.

After that, 0.6 volume of cold isopropanol was added to the microcentrifuge tube and stored at -20°C for 30 minutes to precipitate the DNA. The microcentrifuge tube containing the mixture was then centrifuged at 13,000 rpm at 15 minutes. The supernatants were poured off and the DNA pellets were washed with 1 ml of 70% ethanol at room temperature and each tube was inverted with quick centrifuged at 12,000 rpm for 1 minute. The ethanol was then removed and air-dried. Finally, 20 µl of ultrapure water was added to resuspend the DNA sample (Volume of ultrapure added was depending on the size of DNA pellet).

3.2.3 DNA Purification

The isolated DNAs were purified using Wizard® Genomic DNA Purification Kit (Promega, USA) based on the manufacture's protocol. The DNA samples were topped up to 600 µl with double-distilled water. Then, 3 µl of 4mg/ml RNase A Solution was added into each of the microcentrifuge tube containing DNA. The DNA samples were mixed by inverting the tube 2-5 times. The mixtures were then incubated at 37°C for 15 minutes. Later, the tubes containing mixture were allowed to cool to room temperature for 5 minutes before proceeding.

Next, 200 µl of Protein Precipitation Solution was added into each tube and then mixed gently by inverting the tubes. The tubes were centrifuged for 5 minutes at 13,000 rpm. The supernatant containing the DNA was carefully removed and transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol. The solution was gently mixed by inversion for 30 minutes at room temperature until thread like structure of DNA form a visible mass.

The tubes were centrifuged at 13,000 rpm for 10 minute at room temperature. The supernatant was decanted and 600 µl of room temperature 70% ethanol was added. The tubes were gently inverted several times to wash the DNA. The tubes were centrifuged at 13,000 rpm for 1 minute. The supernatant was decanted. The pellet was air-dried and ddH_2O was then added to resuspend the DNA. The quality and quantity of the purified DNA were then analyzed on 0.8% agarose gel.

3.3 DNA Quantification

The purified DNA was quantified using Lambda 25 UV/VIS Spectrophotomer (Perkin Elmer, USA). Three microlitres of purified DNA from each sample were transferred into quartz cuvette and then diluted with 2997 μl of double distilled water with the dilution factor of 1000×. Later, the absorbance readings were measured at wavelength of 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}).

The absorbance ratio and concentration of each DNA sample was calculated. The DNA amount was further quantified using the agarose gel electrophoresis method to confirm the readings obtained. The DNA concentration was calculated by comparing the band intensity of the DNA samples with the band intensity of the λ *Hin*d III marker.

The mass of λ *Hin*d III DNA in a selected fragment

$$= \frac{\text{Fragment bp} \times (\text{Concentration of } \lambda \text{ DNA}) \times \text{Volume of } \lambda \text{ DNA})}{\text{Total } \lambda \text{ bp}}$$

Concentration of DNA samples

$$= \frac{\text{mass of } \lambda \textit{Hin}\text{d III DNA in a selected fragment}}{\text{Volume of sample DNA}}$$

Table 3.1: Mass of λ *Hind* III DNA in a selected fragment

Band (bp)	≈ng DNA
23,130	143.07
9,416	58.24
6,557	40.56
4,361	26.97
2,322	14.36
2,027	12.54
564	3.49
125	0.77
Total= 48,502	

3.4 Primer design

The following criteria were used for primer design: primer length of 20-25 bp, GC content 40-60%, and T_m between 57-63°C. In addition, the 3'-end of primers optimally consist of one or two Cs or Gs, this is important to prevent "breathing" of ends and increases efficiency of priming. Moreover, a primer should not contain sequences that allow hairpin formation and/or base pairing with itself or the complementary primer. Primers were synthesized by Bio Basic Inc (Canada).

First primer pair was designed from cDNA sequence of *CCR* from *N. cadamba* using sequence Ncdx036.B04 of 699 bp (obtained from Cadamomics) and Primer Premier 5.0 software. The designed primer pair consists of the forward primer with sequence 5' AAGTTGTCCCCCGCCGTCAG 3' and reverse primer with sequence 5' TCACCACTAAGTCCACTCCTTTCTC 3'. This primer pair generated the partial sequence of *CCR* with size of 463 bp. The PCR product was then continued with cloning and DNA

sequencing at Step 3.6 and Step 3.7 respectively to verify the amplified product of *CCR* gene from *N. cadamba*.

Second primer pair was designed because the amplified product of *CCR* gene from *N. cadamba* using the first primer pair did not show homology after checked by using BLASTn (Altschul *et al.*, 1990) through BLAST Search Engines (http://blast.ncbi.nlm.nih.gov/). The second primer pair was designed from cDNA sequence of *CCR* from *N. cadamba* using sequence Ncdx036.B04 of 699 bp (obtained from Cadamomics) and Primer 3.0 software (available at URL: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The designed primer pair consists of the forward primer with sequence 5' CCCGACGATGCAAAGAATAG 3' and reverse primer with sequence 5' AGAATGTGTGCCAATGCAAC 3'. This primer pair was estimated to generate the partial sequence of *CCR* with size of 581 bp.

3.5 Polymerase Chain Reaction (PCR)

PCR reaction mixture includes 30 ng of DNA template, 5 pmol of forward and reverse primers, 0.2 mM dNTPs, 1× PCR buffer (200 mM Tris-HCl pH 8.8, 100 mM KCl, 1% Triton X-100, 100 mM $(NH4)_2SO_4$, 1 μg mL^{-1} BSA), 1.5 mM $MgCl_2$, 1 U *Taq* DNA Polymerase (Promega, USA) and sterile distilled water to make up 25 μl.

CCR amplification for primer set 1 was done in Mastercycler Gradient Thermal Cycler (Eppendorf, Germany) for 2 min at 95°C, 35 cycles of 45 sec at 94°C, 45 sec at 49.6°C and 1 min at 72°C, followed by final extension of 10 min at 72°C. On the other hand, *CCR* amplification for primer set 2 was done in Mastercycler Gradient Thermal Cycler (Eppendorf, Germany) for 2 min at 95°C, 35 cycles of 45 sec at 94°C, 45 sec at 65°C and 1 min at 72°C,

followed by final extension of 10 min at 72°C. The PCR products were then examined on a 1.5% agarose gel and 100bp DNA ladder (BioLabs, New England) was run simultaneously.

3.6 Cloning

3.6.1 Purification of DNA from Agarose Gel

Purification of DNA from agarose gel was performed through QIAquick® Gel Extraction Kit, QIAGEN, Germany. The PCR product was excised and transferred into pre-weight microcentrifuge tube. The tube that contained the gel fragment was weighted again to obtain the amount of gel. Then, 3 volumes of Buffer QG was added to 1 volume of gel and incubated at 50°C for 10 min or until the gel is fully dissolved. Later, 1 gel volume of isopropanol was added and mixed by flicking. A QIAquick spin volume was placed in a provided 2 ml collection tube. The DNA mixture was then pipetted into the column and centrifuged for 1 min.

After that, the flow through was discarded and the column was placed back to the collection tube. Next, 750 μl of Buffer PE was added into the column and centrifuged for 1 min. The flow through was discarded and centrifuged again for 5 min. then, a QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. Then, 50 μl of distilled water was added to the centre of the membrane and the column was centrifuged for 1 min. Finally, the DNA was examined on a 1.5 % agarose gel and λ *Hin*d III DNA marker (Promega, USA) was run simultaneously to determine DNA concentration.

3.6.2 DNA Ligation